Award Number: W81XWH-10-1-0338

TITLE: Investigating the Role of HOXC10 as a Mediator of Metastasis in Breast Cancer

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REPORT DATE: October 2013

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
October 2013	Annual Summary	15 September 2012- 14 September 2013

4. TITLE AND SUBTITLE

Investigating the Role of HOXC10 as a Mediator of Metastasis in Breast Cancer

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-10-1-0338

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

The overall purpose of this project is to investigate the role of HOXC10 in breast cancer tumorigenicity and drug resistance. Since it was previously shown to be involved in proliferation and cell cycle, we investigated at the molecular level the role of HOXC10 and found that by affecting the RB/E2F1 pathway, it controls proliferation, G1/S transition and new origin firing. On the other hand, HOXC10 activates NF-kb, G2/M checkpoint and DNA repair through NER pathway, protecting cells from apoptosis and DNA damage, especially DNA crosslinks. This eventually leads the cells to become less sensitive to chemotherapy treatment. Mechanistically, HOXC10 binds to CDK7 and stimulates its kinase activity towards RNA polymerase II after DNA damage, allowing the cells to finish their repair and to recover from DNA damage arrest by restarting their transcription and protection from apoptosis. Consequently, inhibiting CDK7 could restore chemosusceptibility. Finally, high HOXC10 expression is correlated with poor outcome and with chemoresistance in breast cancer patients and cell lines.

15. SUBJECT TERMS
HOXC10, breast cancer, apoptosis, DNA repair, CDK7, chemo-resistance, BS-181, poor outcome

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	15	19b. TELEPHONE NUMBER *(include area code)*

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Introduction:

HOX genes have been well described as important players in development and morphogenesis, and more recently, in carcinogenesis. Work from our lab and others have shown that some of these genes have important role in breast cancer progression and metastasis, since they control processes such as angiogenesis, DNA repair, apoptosis, and migration. To better identify the key HOX players in breast cancer, we conducted a HOX tiling array, and found that HOXC10 was among the most significantly overexpressed genes in primary and metastatic breast tumors compared to normal tissue. In this proposal, we are addressing the function of HOXC10 in breast cancer progression and drug resistance.

Body:

Task1. To determine the transforming effect of HOXC10 overexpression in cancer breast cell lines using in vivo and in vitro approaches

Summary of previously reported data:

HOXC10 was overexpressed by at least 10 fold in 67% of invasive breast carcinomas, and in 82% of distant metastatic samples, as shown by qRT-PCR analysis of primary and metastatic tissues. This upregulation is functional and increases the tumorigenicity of breast cancer cells, as shown by:

1- Stably overexpressing or knocking-down HOXC10 levels in some breast cancer cell lines affected invasion, anchorage-independent growth and proliferation, especially under low growth factor conditions in vitro. When assessed in vivo, xenografts with high HOXC10 expression grew faster, were more vascularized and expressed elevated levels of chemokines and cytokines.

2- HOXC10 increases proliferation by facilitating G1/S transition, S progression and replication resumption. This function is accomplished by indirectly activating the oncogenic E2F1 pathway and its proliferative signature. It also requires the homeodomain motif of HOXC10, and its ability to bind to DNA.

Task2. To determine the downstream pathway(s) through which HOXC10 functions to decrease response to chemotherapy treatment

Summary of previously reported data:

HOXC10 activates EGFR, E2F1 and PI3K/AKT pathways, which are important for cancer cell survival. This led us to investigate if HOXC10 affects response to chemotherapy treatment and we therefore found that cell lines with high HOXC10 expression (endogenous or exogenous) have reduced sensitivity to many drug classes, including the anthracyclins, platins, taxanes and nucleoside analogs. HOXC10 increases resistance to these drugs by protecting cells from apoptosis, activating NF-kB, and enhancing the late steps in DNA damage repair, mainly through the nucleotide excision repair (NER) pathway.

Progress:

The following in-vitro experiments were conducted in 2 systems: 1- in MCF10A-Ras overexpressing either an empty vector (v) or HOXC10; 2- in SUM159 expressing either a scramble shRNA (scr) or a shRNA specific for HOXC10 (knockdown efficiency 50-70%).

HOXC10 is involved in DNA crosslinks repair and checkpoint recovery.

A. To confirm that HOXC10 is important in the repair of bulky DNA crosslinks and adducts, we exposed our cells to UV light, and allowed them to recover and grow as colonies. As represented in Figure 1A and quantified, cells that expressed high levels of HOXC10 (10A-Ras-C10 and SUM159-scr) were able to survive better the increase doses of UV.

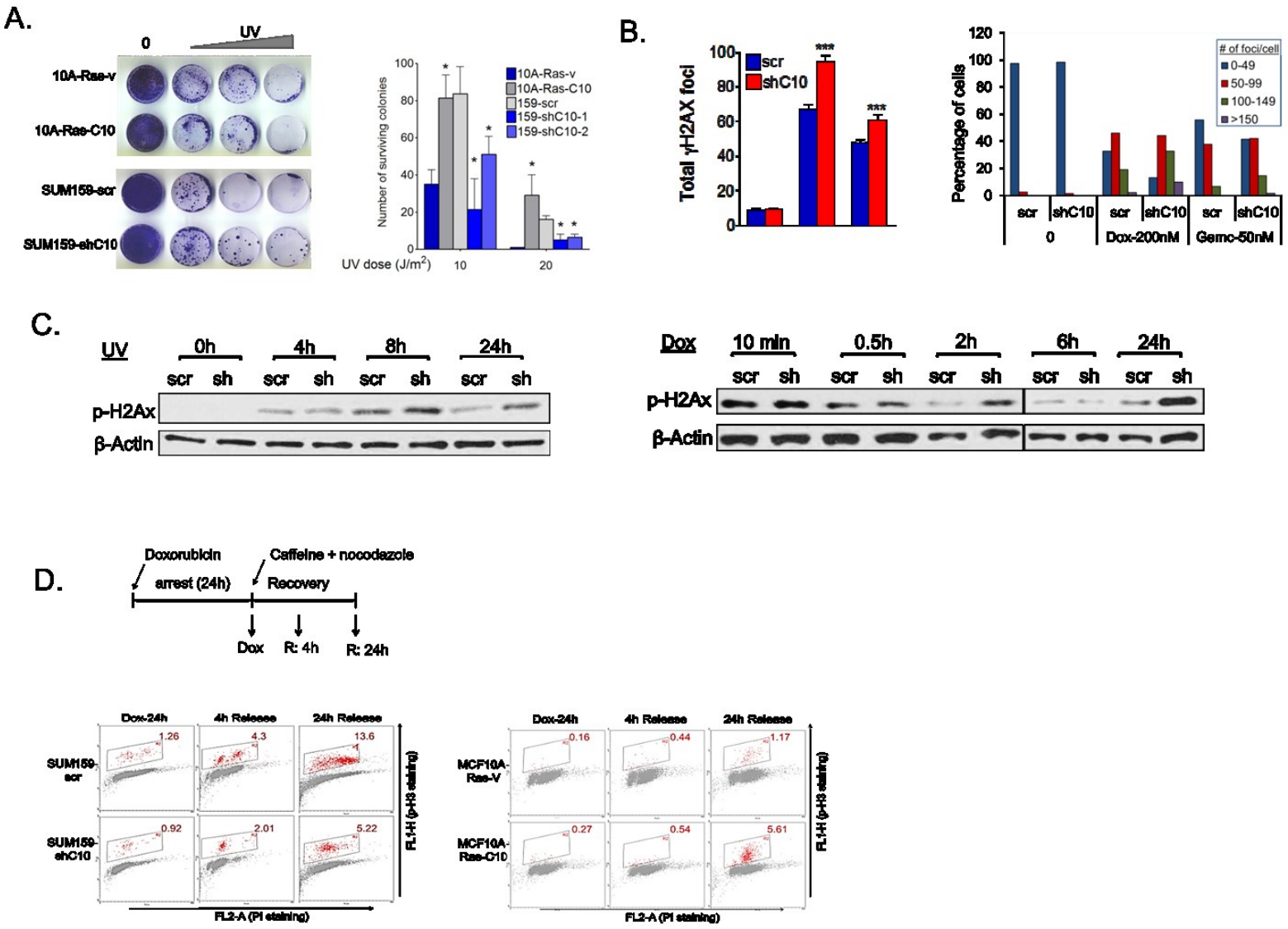
B. We next studied the phosphorylation of histone H2AX, the surrogate marker of DNA repair efficiency by NER by quantifying the number of residual γH2AX foci after 24h of initial treatment (immunofluorescence). There were on average 20-30% more foci accumulating in SUM159-shC10 after chemotherapy treatment (left) and with almost 50-60% more cells displaying at least 100 foci/nucleus (right) (Figure 1B)

C. Interestingly, time-course analysis showed no differences in the initial induction of phospho-H2AX (10min-8h) after UV or chemotherapy (Figure 1C), in accordance with reports showing that the residual γH2AX measured 24 h after treatment- and not the initial kinetics of γH2AX formation- was a better predictive of cell viability (1,2).

D. Upon DNA damage, the cell cycle checkpoints are activated to halt the progression of cells until repair is complete, preventing therefore apoptosis and mitotic catastrophe. Checkpoint recovery after DNA damage is a key pathway that allows cancer cells to escape the arrest and to continue proliferation after DNA damage. We therefore wondered if HOXC10 was required for cell-cycle re-entry following DNA-damage-induced arrest. We therefore temporary treated cells with doxorubicin, and then we monitored the percentage of cells that re-entered mitosis after caffeine addition. As Figure 1D shows, in the absence of HOXC10, cells were defected in restoring their growth.

These results confirmed that HXOC10 activates the repair of DNA crosslinks when the cells arrest, and then accelerates checkpoint recovery to allow cells to resume their growth.

Figure 1.



A. HOXC10 effectively protects cells from UV exposure. Representative image of the colony survival assay and its quantification after 7 days of initial UV exposure. B. Cells were treated with doxorubicin or gemcitabine for 24h. γH2Ax foci were stained and quantified using ImageJ software. Average number of foci in >150 nuclei is shown in the left graph. Percentage of cells with the noted numbers of γ-H2AX foci is presented on the right and shows that more cells with >150 foci accumulate in the knockdown cell line. C. SUM159-scr and –shC10 were treated with 200nM doxorubicin or with UV light. Protein was extracted at the indicated time, and the kinetics of H2Ax phosphorylation was monitored by western blot. D. Scheme of the recovery-induced experimental setting. Cells were collected at the indicated time, and the amount of mitotic cells was determined by pH3 positivity by FACS. All experiments were done in triplicate. * $p<0.05$, *** $p<0.0001$, t-test analysis).

HOXC10 binds to CDK7 during chemotherapy treatment.

A recent publication lists HOXC10 as one of the binding partner of CDK7, linking it to RNA polymerase II during transcription (3). Since CDK7 drives DNA repair, NF-kB activity, proliferation and protection from apoptosis, we hypothesize that HOXC10 promotes chemoresistance by binding to CDK7.

A. After overexpressing exogenous HOXC10 in 293T, cells were treated with different drugs and then protein complexes were co-immunoprecipitated (co-IP) with anti-CDK7. HOXC10 weakly co-IP with CDK7 (Figure 2A). However, upon DNA damage with different chemotherapeutic drugs, the fraction of interaction increased dramatically. Further, CDK7 activity was required for this binding: inhibiting its activity using the pharmacologic inhibitor SNS-032, or by expressing the dominant negative, kinase-dead mutant CDK7 (D155A) reduced its interaction with HOXC10 after doxorubicin treatment (Figure 2A and Figure 2B).

B. This interaction preferentially activates CDK7 activity towards phosphorylating RNA polymerase II (RNA polII), as evident by a decrease binding of CDK7 to RNA polII (co-IP, Figure 2C), a decrease in its kinase activity (Figure 2D), and a drop in the expression of its target gene MCL1 (Figure 2E) when HOXC10 is knockdown in SUM159 cells by 2 independent shRNA (shC10-1, shC10-2).

Targeting CDK7 in HOXC10 overexpressing cells can reverse their chemotherapy resistance.

Given that the interaction of CDK7 and HOXC10 can mediate drug resistance of the breast cancer cell lines, we hypothesize that targeting CDK7 can reverse HOXC10 phenotype. We decided to test this model and use known CDK7 inhibitors, as a more efficient approach to inhibit HOXC10 function than the proposed drug screening experiment (Aim 2c in the grant).

A. First, using siRNA, we reduced CDK7 protein levels by around 60%-80% in SUM159 cell line system, and then treated cells with different drugs (Figure 3A). After decreasing CDK7 levels, cells not only slowed down their growth, but also responded similarly to drug treatment, confirming our hypothesis.

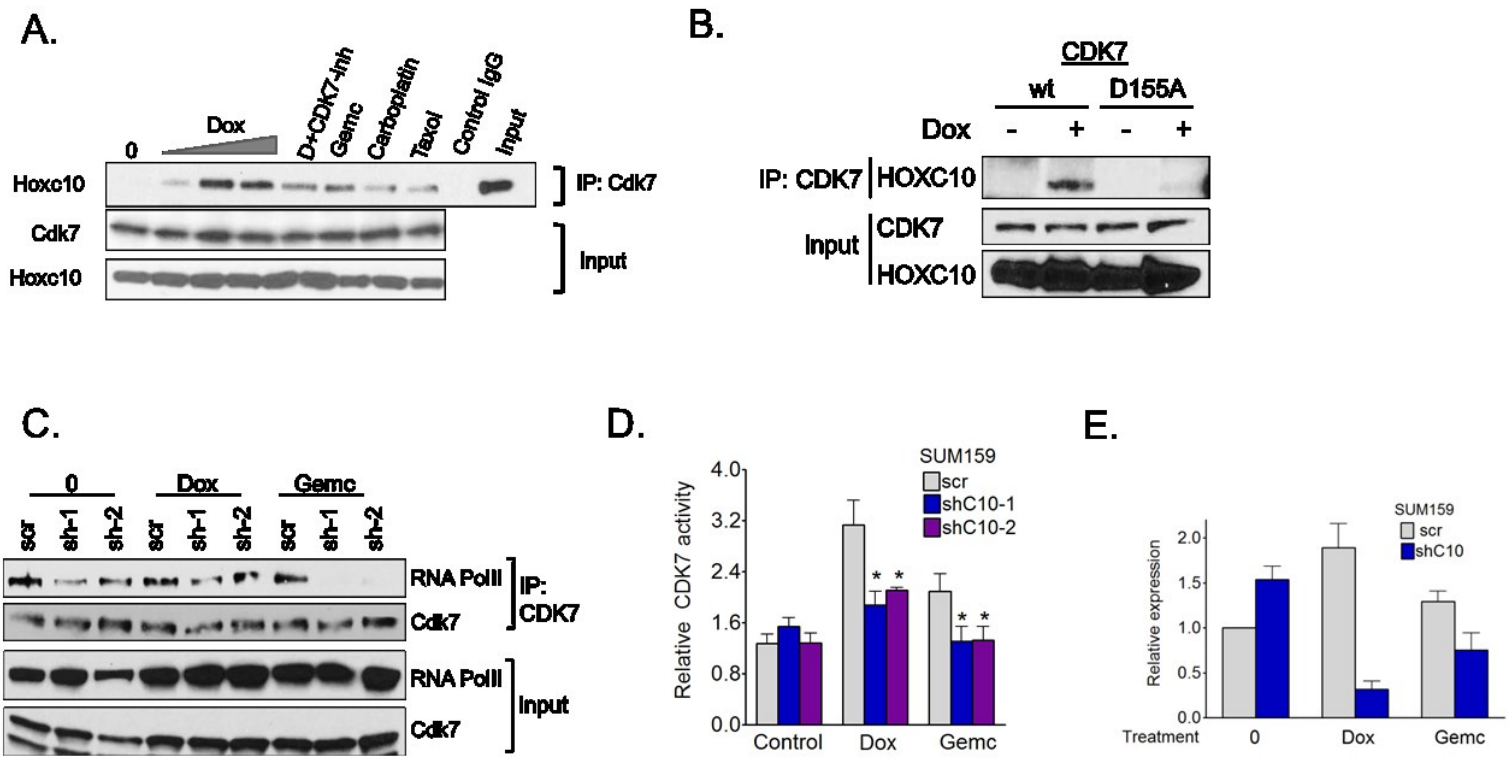
B. Many CDK7 inhibitors have been recently developed, including the selective inhibitor BS-181 (4) and SNS-032 (5). Adding CDK7 inhibitors to different chemotherapy drugs led to a better response (Figure 3B). Interestingly, the level of response of SUM159-scr with the co-treatment was equal to that of SUM19-shC10 when treated with chemotherapy alone. This result highlights that the activation of CDK7 is the major mechanism through which HOXC10 drives survival after chemotherapy treatment.

C. To confirm these data, we used Taxol (Tax-R) and Epirubicin (Epi-R) MCF7 resistant sublines. These cells were established by a progressive exposure to a drug (epirubicin or paclitaxel), and display 815 and 535 fold increase in their resistance, respectively (6). Co-treatment with BS-181 along with cytotoxic drugs restored chemo-susceptibility of these

cells, as shown by MTT and colony survival assays (Figure 3C, 3D), strengthening the usefulness of targeting CDK7 to reverse chemoresistance.

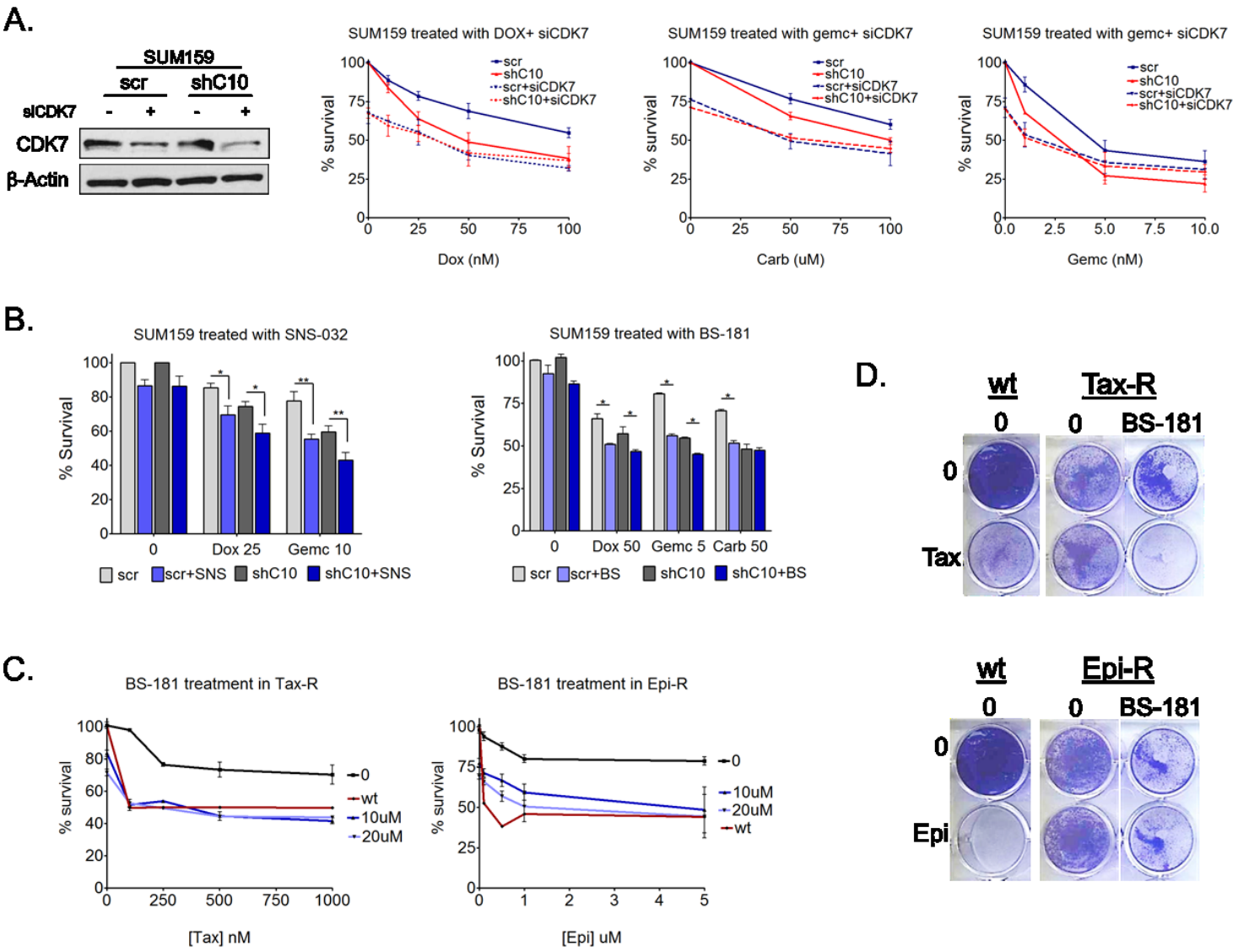
In sum, HOXC10, by interacting with CDK7, activates DNA repair (mainly NER pathway), checkpoint recovery, resumption of transcription after DNA damage, and protection of apoptosis. This eventually leads to resistance to chemotherapy treatment. Since no known inhibitors for HOXC10 is known, a promising strategy to reverse this chemoresistance is through direct inhibition of CDK7.

Figure 2.



A. 293T cells were transfected with HOXC10 vector and then treated with different drugs for 24h. Cell lysates were co-IP with CDK7 (D: Doxorubicin; CDK7-inh: SNS-032). B. HOXC10 and either CDK7 wt or its kinase mutant (D155A) were coexpressed in 293T and treated with doxorubicin for 24h. Binding of HOXC10 to CDK7 was assessed by co-IP with CDK7. C. Stable cell lines were treated with dox or gemcitabine for 24h and cell lysates were IP with CDK7. CDK7 association to TFIIH during DNA damage- as detected by RNA Pol II binding- is decreased in the absence of HOXC10. D. The kinase activity of CDK7 towards a recombinant GST-CTD was assessed 24h after treatment of SUM159 with 200nM dox or gemc. This activity is reduced after HOXC10 kd. E. Level of MCL1 (marker of CDK7 activity) was assessed by RT-PCR in the SUM159 system (* p,0.05, t-test).

Figure 3.



A. CDK7 was inhibited with siRNA in SUM159-scr and –shC10. 24h later, cells were treated with doxorubicin, gemcitabine or carboplatin. MTT assay was performed after 48h. The western blot shows a decrease of at least 70% in the protein levels of CDK7 with the siRNA transfection. B. Cells were co-treated with chemotherapy and with 2 different CDK7 inhibitors, SNS-032 (left) or BS-181 (right). MTT assay was performed after 48h. Both A and B show that chemo-susceptibility is restored upon CDK7 inhibition. C. Taxol or Epirubicin resistant MCF7 were treated with taxol or epirubicin respectively along with different concentrations of BS-181. MTT was conducted 48h later. D. Resistant cells were treated with 200 nM taxol or 500 uM epirubicin alone or with combination with 20uM BS-181. Cells were allowed to grow for 7 days, then surviving colonies were stained with crystal violet. All data were repeated independently 3 times, and anaylzed by t-test (* $p<0.05$, ** $p<0.001$).

Task3. To determine the value of HOXC10 expression as a prognostic and diagnostic marker

Summary of previously reported data:

Mining the online databases like “Oncomine” and “NexBio” revealed that HOXC10 was always among the 1% genes overexpressed in breast cancer, regardless of the grade or subtype. High levels of HOXC10 were also found in resistant cell lines, established by continuous chemotherapy treatment either in-vitro or in-vivo.

Progress:

A. HOXC10 expression is prognostic in chemotherapy treated patients (n=548, HR=1.7, p=0.009), but not in all patient (n=3005, p=n.s.) for relapse-free (Figure 4A) or overall survival ((Figure 4B) in samples downloaded from GEO in which Affymetrix microarrays were used.

B. HOXC10 is also prognostic for overall survival in chemotherapy treated patients in the METABRIC cohort which uses Illumina microarrays (n=419, HR=1.5, p=0.016) (Figure 4B, right).

C. This significance is retained (p=0.00014) in Cox multivariate regression analysis including known clinical parameters (HOXC10, ER, HER2, lymph node status, grade, age and MKI67 expression) (Figure 4C).

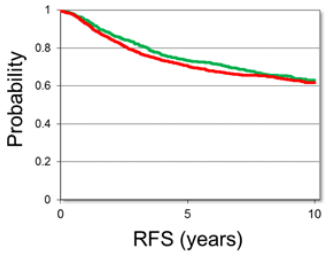
D. When using a ROC analysis, HOXC10 was predictive for response to anthracycline-taxane based chemotherapy regimens (n=974, p=4.7e-04, AUC=0.571) (Figure 4D).

All these data highlights that HOXC10 correlates with poor prognosis in breast cancer treated patients.

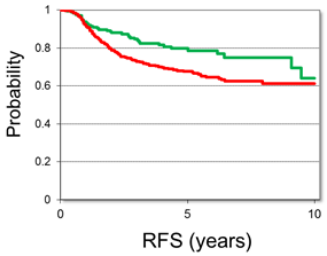
Figure 4. A-B. Kaplan-Meier analysis was performed by employing an updated version of the online available KM-plotter using 3,999 patients http://kmplot.com/analysis. High HOXC10 levels was significantly associated with worse relapse free survival (RFS) and OS in chemotherapy treated patients, but has no prognostic significance in all patients. C. A Cox multivariate regression analysis was made for all patients and for chemotherapy treated patients for HOXC10, ER, HER2, lymph node status, grade, age and MKI67 expression. D. Receiver operating characteristic (ROC) analysis was performed in the R statistical environment to assess the predictive role of HOXC10. Statistical significance was set at $p<0.01$.

A.

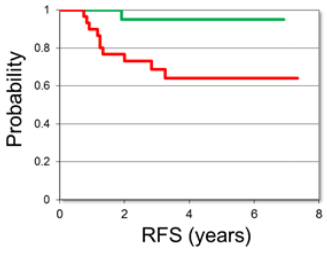
All patients (n=3005, HR=1.1, p=0.11)

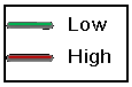


Chemotherapy treated patients (n=548, HR=1.7, p=0.009)



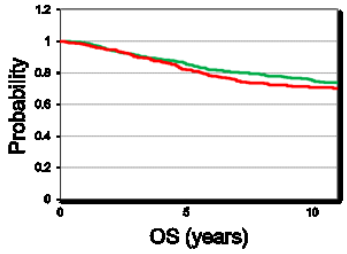
RFS in patients treated with taxanes + anthracyclines (n=50, HR>5, p=0.017)



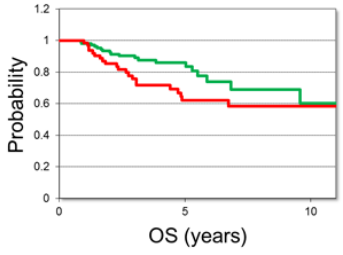


B.

All patients (n=1112, HR=1.19, p=0.18)

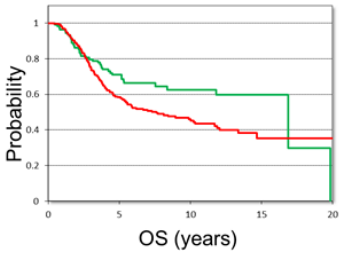


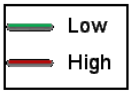
Chemotherapy treated patients (n=528, HR=1.4, p=0.067)



<u>Metabric dataset</u>

Overall survival in chemotherapy treated patients (n=419, HR=1.5, p=0.016)



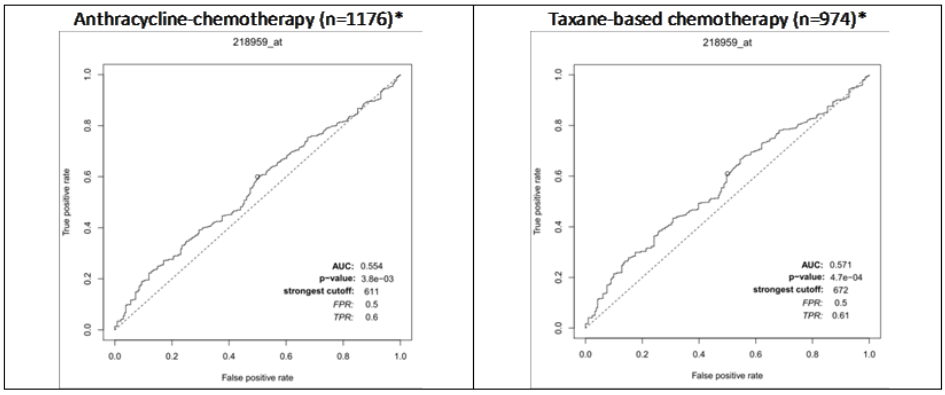


C.

<u>Cox regression in chemotherapy treated patients</u>

	Coefficient	95% Conf. (±)	Std.Error	P	Hazard = Exp(Coef.)
218959_at	0.000214095	0.000130885	6.67784E-05	0.001345973	1.000214118
ESR1 expression on array	-6.4378E-05	4.7593E-05	2.42823E-05	0.008019912	0.999935624
HER2 expression on array	-2.50816E-06	2.33633E-05	1.19201E-05	0.833345139	0.999997492
Lymph node status	0.637741507	0.507754195	0.259059885	0.013826036	1.892202523
Grade	0.288681801	0.367781043	0.187644564	0.123938614	1.334666972
Age	0.022281756	0.016154682	0.008242236	0.006864325	1.022531849
MKI67 expression on array	0.000163305	0.000701688	0.000358006	0.648280437	1.000163319

D.



Key Research Accomplishments:

- HOXC10 is commonly overexpressed in breast cancer.
- HOXC10 is a novel oncogene stimulating proliferation and tumorigenicity of the cells, at least through activating E2F1 pathway and facilitating G1/S transition and S phase progression.
- By decreasing apoptosis, activating NF-kB pathway, enhancing DNA repair pathway (mainly NER pathway) and stimulating checkpoint recovery, HOXC10 decreases susceptibility of the cells to chemotherapy treatment.
- HOXC10 binds to CDK7 after drug treatment and stimulates its activity towards RNA PolII, leading to chemoresistance.
- Targeting CDK7 is a promising strategy to reverse the function of HOXC10 and restore chemo-susceptibility.
- HOXC10 is upregulated during acquired resistance in-vitro and in-vivo.
- HOXC10 correlates with poor prognosis in chemotherapy-treated breast cancer patients.

Reportable Outcomes:

- Degree
  PhD in Cellular and Molecular Medicine at Johns Hopkins University, School of Medicine

- Abstract
  1- Sadik H, Nguyen Nguyen, Rakesh Kumar, Tej Pandita, Sukumar S (December 2012). HOXC10, a Homeobox protein Overexpressed in Breast Cancer, modulates the response to Chemotherapy treatment. San Antonio Breast Cancer Symposium (SABCS), San Antonio, TX
  2- Sadik H, Nguyen N, Nilay Shah, Rajesh A. Gupta, Howard Y. Chang, Sukumar S (August 2011). HOXC10, a Homeobox protein Overexpressed in Breast Cancer, modulates the response to Chemotherapy treatment. DoD Breast Cancer Era of Hope, Orlando, FL.
  3- Sadik H, Nilay Shah, Rajesh A. Gupta, Howard Y. Chang, Sukumar S (December 2010). The homeobox protein HOXC10 is overexpressed in breast cancer and confers resistance to chemotherapy. San Antonio Breast Cancer Symposium (SABCS), San Antonio, TX.

Conclusion:

As a clear involvement of the HOX family in carcinogenesis has been accumulating over the years, we took a high-throughput approach on the HOX loci and found that HOXC10 is one of the most significantly overexpressed genes in breast tumors as compared to normal tissues. This proposal addresses for the first time the function of HOXC10 in breast cancer development and drug resistance.

First, HOXC10 increases tumoreginicity and proliferation of breast cancer cell lines, both in vitro and in vivo. Unlike other reports suggesting a function of HOXC10 during G2/M transition, I found that HOXC10 promotes continuous growth by facilitating G1/S transition and S phase progression, and by activating E2F1, a key protein in this cell cycle phase.

A more interesting outcome of my work has been through finding that HOXC10 overexpressing cells were more resistant to some chemotherapeutic drugs used commonly in breast cancer treatment. HOXC10 activates NF-kB allowing the cells to be less prone to apoptosis, enhances DNA repair, mainly the NER pathway and allows cells to recover from their checkpoint arrest once their repair is completed. Mechanistically, HOXC10 interacts with CDK7 after DNA damage, enhancing its binding and activity towards RNA Polymerase II. This allows the cells to complete their repair and restart their transcription for complete recovery and for protection of apoptosis.

Importantly, I found that by using CDK7 inhibitors, chemosusceptibility in breast cancer can be restores the effect of HOXC10 overexpression can be reversed. As there are no known HOX inhibitors, and as CDK7 inhibitors are already in clinical development, targeting CDK7 might be a promising strategy to overcome chemoresistance in breast cancer.

Lastly, the more direct outcome of my work is by finding that HOXC10 can be used as a prognostic marker. Higher expression of HOXC10 is correlated with poor prognosis in chemotherapy treated patients, and with increase resistance to treatment, even from the early phases.

Training:

During the reported period, Dr Sukumar along with my thesis committee were monitoring my progress and guiding me through completion of the tasks of my thesis and my grant. I also had the opportunity to attend different conferences for breast cancer and present posters. Weekly meetings and conferences (every Tuesday, Wednesday and Friday) from scientists at Hopkins or outside were of great value. Further, I am having great opportunities to collaborate with experts in DNA damage experts in and outside Johns Hopkins University. Also, all the research facilities are provided within a walking distance and are available all the time. Finally, the lab environment is so rich with post-docs and pre-docs with different skills and background, which helped me master basic methods and more complicated techniques such as flow cytometry and in vivo work. In sum, inside and outside supports are continuously available for me to complete the task of my grant successfully.

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Appendices:

Sadik H, Nguyen Nguyen, Rakesh Kumar, Tej Pandita, *Sukumar S* (December 2012). HOXC10, a Homeobox protein Overexpressed in Breast Cancer, modulates the response to Chemotherapy treatment. *San Antonio Breast Cancer Symposium (SABCS)*, San Antonio, TX

Background: Breast cancer is the second leading cause of cancer deaths in women worldwide. Although chemotherapy is effective, resistance to drugs develops over time and can account for treatment failure in over 90% of metastatic breast cancer patients. HOX genes are homeobox-containing transcription factors well-known for their role in morphogenesis. However, accumulating evidence has emphasized their importance during carcinogenesis and metastasis. The goal of this study is to understand the role of HOXC10 in breast cancer and the consequence of its overexpression in the response to chemotherapy.

Methods: Using a tiling array of all four HOX clusters in a panel of primary and metastatic breast cancer tissues, we identified HOXC10 as being among the highly overexpressed genes in breast cancer. Then using a panel of cell lines that either stably overexpress exogenous HOXC10 or cell lines with stably downregulated endogenous HOXC10 (mediated by shRNA), we investigated the role of HOXC10 in proliferation, response to chemotherapy treatment and repair of DNA damage.

Results: HOXC10 is overexpressed in 67% of primary breast tumors (n=31), in 82% of the metastatic tissues (n=49) and in most breast cancer cell lines (n=48). In vitro and in vivo investigation confirmed that HOXC10 plays an oncogenic role in breast cancer. Further, knockdown of HOXC10 in a panel of breast cancer cell lines slowed their proliferation and arrested them at the G1 phase, by inactivating the RB/E2F pathway, decreasing the number of new origins and eventually reducing the polyploidy population.

Cell survival assays after different chemotherapeutic drug treatment showed that overexpression of the exogenous HOXC10 in MCF10A led to less susceptibility to most drugs. This was partially due to a protection from apoptosis by upregulating and activating the anti-apoptotic machinery such as the NF-kb pathway. Further investigation revealed the involvement of HOXC10 in DNA repair (and not initial response), especially after DNA crosslink damage. Interestingly, the binding of HOXC10 to CDK7 in a region outside its homeodomain activates CDK7 activity towards RNA polymerase II mainly in response to DNA damage. Since HOX genes are difficult to target therapeutically, one potential approach to overcome chemoresistance in HOXC10 overexpressing cells is by including CDK7 inhibitors (already in clinical trials).
All these results were confirmed in the SUM159 model which stably expresses a HOXC10-shRNA.

Finally, HOXC10 was found to be significantly overexpressed in MCF7 isogenic cell lines gradually selected to be resistant to some chemotherapeutic drugs. By knocking down HOXC10 in these sublines, resistance to the drug was reduced. Further, SUM159 and MDAMD231 xenografts that were treated with chemotherapy over weeks and that show partial to no response tend to have a higher expression of HOXC10.

Conclusion: This study shows that HOXC10, a homeobox protein previously shown to be regulated during the cell cycle and to have a positive effect on proliferation, is overexpressed in the majority of breast cancers. This upregulation may have clinical implications since cells with higher expression of HOXC10 tend to have more genomic instability and activation of anti-apoptotic and DNA repair pathways, which eventually modulate the response to some chemotherapy drugs.